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(54) Title: POLYMORPHISM II: LINKAGE OF ASTHMA TO A LOCUS ON CHROMOSOME 4

(57) Abstract

A method for diagnosing an individual as being asthmatic, or as having a predisposition to asthma is described, which method comprises demonstrating in the individual the presence or absence of one or more alleles which are associated with asthma, wherein the one or more alleles are situated at a locus in a region of chromosome 4 of up to 1 megabase in length, which region contains the locus D4S3032 and/or D4S2921.

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POLYMORPHISM II: LINKAGE OF ASTHMA TO A LOCUS ON CHROMOSOME 4

This invention is concerned with methods for the diagnosis of asthma and with materials and methods relating thereto.

Asthma is a disease which is becoming more prevalent and is the most common disease of childhood (1). Most asthma in children and young adults is initiated by IgE mediated allergy (atopy) to inhaled allergens such as house dust mite and cat dander. However, not all asthmatics are atopic, and most atopic individuals do not have asthma, so that factors in addition to atopy are necessary to induce the disease (2,3). Asthma is strongly familial, and is due to the interaction between genetic and environmental factors. The genetic factors are thought to be variants of normal genes ("polymorphisms") which alter their function to predispose to asthma.

Asthma may be identified by recurrent wheeze and intermittent air flow limitation. An asthmatic tendency may be quantified by the measurement of bronchial hyper-responsiveness in which an individual's dose-response curve to a broncho-constrictor such as histamine or methacholine is constructed. The curve is commonly summarised by the dose which results in a 20% fall in air flow (PD20) or the slope of the curve between the initial air flow measurement and the last dose given (slope). Asthma is accompanied by blood eosinophilia, and eosinophils are prominent in asthmatic airways.

In the atopic response, IgE is produced by B-cells in response to allergen stimulation. These antibodies coat mast cells by binding to the high affinity receptor for IgE (FcεRI). When a multivalent allergen binds to an IgE-coated mast cell, the cross-linking of adjacent IgEs by allergen initiates a series of cellular events leading to the destabilisation of the cell membrane

and release of inflammatory mediators. This results in mucosal inflammation, wheezing, coughing, sneezing and nasal blockage.

Atopy can be diagnosed by (i) a positive skin prick test in response to a common allergen; (ii) detecting the presence of specific serum IgE for allergen; or (iii) by detecting elevation of total serum IgE.

Genetic factors underlying a disease may be identified through localisation to particular chromosomal regions by genetic linkage. Genetic linkage is established by the study of families. It relies on matching the inheritance of disease with genetic polymorphisms of known localisation (known as "genetic markers"). In a complex disease such as asthma, genetic linkage will typically localise genes to within 10 - 20 Megabases (Mb) of DNA. A region of this size may contain 350 - 700 genes, and will be too large to permit immediate identification of the disease-causing gene.

Closer localisation of disease-causing genes may be accomplished by the detection of associations between particular alleles and the disease phenotype. Over short segments of DNA, distinctive alleles of the individual polymorphisms will show non-random association with alleles of neighbouring polymorphisms. This phenomenon, known as "linkage disequilibrium" occurs over 50-500 Kilobases (Kb) of DNA. Linkage disequilibrium may be detected by the study of individuals as well as by the study of families.

Disease-causing alleles will be in linkage disequilibrium with non-functional polymorphisms from the same chromosomal segment. It is therefore possible to detect allelic association with disease from particular chromosomal segments, without identifying the exact polymorphism and gene underlying the disease state.

The detection of allelic association may therefore give information as to disease susceptibility in a particular individual. Furthermore,

allelic association is indicative of a disease-causing gene being present within 500 Kb of DNA in either direction from the allele (i.e. 1 Mb in total). Such a region may contain only 30 genes, within which the identification of the disease-causing gene is possible.

The presence of linkage disequilibrium also means that other polymorphisms may be anticipated to associate with disease, and that these additional polymorphisms will also be diagnostic of disease susceptibility in particular individuals.

Genetic associations with atopy have been demonstrated. WO 95/05481 discloses that variants of the gene encoding the β-subunit of the high-affinity receptor for IgE (FcεRIβ) are associated with atopy. It teaches a method for diagnosing atopy which is based upon the demonstration of the presence or absence of one of two variants in a specific portion of the DNA sequence of the gene encoding FcεRIβ, located near the commencement of exon 6 of the FcεRIβ gene on chromosome 11. A further variant has also been found in which the unusual variant sequence is in the coding sequence for the C-terminal cytoplasmic tail of FcεRIβ (4).

Tumour Necrosis Factor (TNF) is a pro-inflammatory cytokine that is found in increased concentration in asthmatic airways (5). We have previously shown that polymorphisms within the TNF gene are associated with an increased risk of asthma (6).

The known polymorphisms do not account for all of the genetic factors which predispose to asthma. In particular, asthma is not necessarily an atopic disease. Identification of further genetic polymorphisms linked to asthma will allow the identification of children at risk of asthma before the disease has developed (for example immediately after birth), with the potential for prevention of disease. The presence of particular polymorphisms may predict the clinical course of disease (e.g. severe as opposed to mild) or the

response to particular treatments. This diagnostic information will be of use to the health care, pharmaceutical and insurance industries.

We have previously established linkage of bronchial hyper-responsiveness to chromosome 4 (8). However, this finding is of no use in diagnosis.

It has now been discovered that a genetic polymorphism known as D4S3032*5 on chromosome 4 and a nearby polymorphism known as D4S2921*13 are associated with asthmatic traits. Specifically, D4S3032*5 is associated with bronchial hyper-responsiveness and D4S2921*13 is associated with peripheral eosinophilia, both of these being traits which underlie asthma. The two polymorphisms can therefore be used as diagnostic tools.

The invention therefore provides a method for diagnosing an individual as being asthmatic, or as having a predisposition to asthma, which method comprises demonstrating in the individual the presence or absence of one or more alleles which are associated with asthma, wherein the one or more alleles are situated at a locus in a region of chromosome 4 of up to 1 megabase in length, which region contains the locus D4S3032 and/or D4S2921.

The 1Mb region of chromosome 4 referred to flanks the D4S3032 and D4S2921 loci. Thus, the specific allele D4S3032*5, or D4S2921*13, or other unusual polymorphisms in the region which are associated with asthma, may be the subject of identification in the method according to the invention. Equally two or more such alleles may be the subject of identification, including in particular the combination of D4S3032*5 and D4S2921*13.

Current diagnostic methods involving detection at the nucleic acid level normally comprise the steps of:

(i) obtaining a suitable tissue sample from the individual;
(ii) preparing from the tissue sample a nucleic acid sample;
(iii) analysing the nucleic acid sample for the presence or absence of the relevant nucleic acid sequence, such as a specific allele.

Preferably, an amplification step is performed prior to the analysis, such that the locus at which the allele is situated is amplified. A preferred amplification technique is the PCR, although any suitable method of nucleic acid amplification may be employed.

In further aspects, the invention provides a pair of oligonucleotide primers for amplification of an allele which is associated with asthma, which allele is situated at a locus in a region of chromosome 4 of up to 1 megabase in length, which region contains the locus D4S3032 and/or D4S2921; and an assay kit comprising the pair of oligonucleotide primers.

The specific allele for identification may take the form of microsatellite repeats, which are nucleotide sequences containing short, repeated nucleotide motifs, usually a dinucleotide or a trinucleotide motif. A pair of primers which hybridize under suitably stringent conditions, to sequences at a position on either side of the microsatellite repeats, may be used to amplify the microsatellite repeats by PCR. Differences in the number of repeats are recognised by size differences in the PCR products. An allele which has a specified number of repeats and therefore a known size can thus be identified. D4S3032*5 and D4S2921*13 are examples of such alleles.

The primers employed in the method comprise nucleic acid sequences which are complementary to, or substantially complementary to unique sequences either side of the microsatellite repeats, such that only the relevant polymorphic region of the genome is amplified. The conditions under which the amplification is performed are gauged such that specific

hybridization of the primers to the flanking sequences occurs and non-specific hybridization is avoided. The hybridization conditions are suitably stringent for that purpose. Standard techniques can be used to identify an appropriate set of reaction conditions.

Typically, the PCR products are detected by means of a detectable label attached to one of the PCR primers. Alternatively another form of labeling may be used such as a labeled sequence specific probe which hybridizes to the amplified sequences. The label may be a fluorescent or other label. The PCR products are subjected to size determination, typically involving size-separation for example by gel electrophoresis, and the presence or absence of the allele of interest is determined.

It will be evident that the invention is not limited with regard to the manner in which the presence or absence of the allele of interest is determined. The labeling, detection, separation or any other aspect of the method as described here may be replaced by other suitable known techniques and reagents.

The allele for identification may be an allele other than D4S3032*5 or D4S2921*13 which is in linkage disequilibrium with D4S3032*5 or D4S2921*13 and is associated with asthma. This includes alleles of both functional and non-functional polymorphisms. Functional polymorphisms include polymorphisms within genes, usually within coding sequences of genes. Non-functional polymorphisms are polymorphisms which do not themselves cause the disease.

This invention will now be further described in the Examples section which follows. The Examples are intended to be illustrative and do not limit the scope of the invention in any way.

EXAMPLES

Description of Laboratory Testing

Subjects

Two panels of subjects have been studied.

Panel A consisted of 80 nuclear families sub-selected from an Australian population sample of 230 families (8). The panel contained a total of 203 offspring forming 172 sib-pairs. 12% of the children were asthmatic.

Panel C consisted of 87 nuclear families recruited through a child attending an asthma clinic in the Oxford region. The families contained 216 offspring (148 sibling pairs), of whom 44% were asthmatic.

Phenotyping

Bronchial responsiveness to methacholine was measured as previously described (8): the maximum dose administered was 12 μmol. The slope of the dose-response curve was calculated as (pre-dose forced expiratory volume in one second (FEV1) - last FEV1) ÷ the cumulative dose of methacholine). A constant of 0.01 was added to each measurement, to allow loge transformation when Slope was ≤ 0. Eosinophils in peripheral blood were Coulter-counted and the values $\log_e$ transformed before analysis.

Genotyping

The microsatellite markers D4S3032 and D4S2921 were typed by semi-automated fluorescent methods, as described previously (8). These markers are in close proximity at the telomeric region of the long arm of chromosome 4.

The polymerase chain reaction primer sequences for the markers were as follows:

D4S3032	5' TGA AAT TCT ATT GAC CAA TGA TGT G (SEQ ID NO: 1)
UD4S3032	5' TAG CAC CTG GAT TTA CCA TGA C (SEQ ID NO: 2)
D4S2921	5' TCC TTC AGG AAC TGG TG (SEQ ID NO: 3)
UD4S2921	5' TTA AAA ATC TAC AGA CAA GGG C (SEQ ID NO: 4)

The polymerase chain reaction conditions were as follows: The reaction volumes were 10μl, containing 50ng of genomic DNA, 200mM dNTPs, 1 x NH4+ buffer, 50ng oligonucleotide primers (forward labelled fluorescently), 0.5 to 3.0mM $MgCl_2$ and 0.2U Taq polymerase. Cycling conditions were 1 min at 95°C, 1 min at 55°C and 45s at 72°C; 28 cycles were used. PCRs were performed on an Hybaid Omnigene thermal cycler.

Electrophoresis and allele scoring were as follows:

PCR products were mixed with a size standard (GS350 TAM) in loading buffer (80% (v/v) formamide, 20% (vlv) 50mM EDTA, 0.1% (w/v) blue dextran). Samples were denatured at 95°C for 4 min immediately prior to loading onto a 6% polyacrylamide gel and were electrophoresed at 800v for 6h on an Applied Biosystems (ABI) 313 DNA sequencer. Allele sizes were assigned using the ABI GENESCAN and ABI GENOTYPER software.

Association Analysis

Association was tested against the phenotype of bronchial hyper-responsiveness and peripheral blood eosinophil counts by the ASSOC routine of the SAGE (ver2.2) computer program.

Results

Association with D4S3032 allele 5 and D4S2921 allele 13

Each of the markers was then tested for association with the asthma phenotype. Association was seen in panel A for allele 5 of D4S3032

(D4S3032*5) and bronchial hyper-responsiveness. This allele is 145 base pairs in size, using the primers described above. Association between allele 13 of D4S2921 (D4S2921*13) and eosinophil counts were seen in both panels. This allele is 162 base pairs in size, using the primers described above. (Other suitable primers can be designed and their amplification product size determined for D4S3032*5 or D4S2921*13, using known sequence information (9).) The results of testing were as follows:

			Panel A		Panel C		Combined	
Trait	Marker	Allele	χ^2	p	χ^2	p	χ^2	p
Slope	d4s3032	5	13.56	0.0002	-	-	-	-
Eosinophils	d4s2921	13	5.00	0.03	11.17	0.0008	17.61	0.0000

The recombination fraction between D4S3032 and D4S2921 was 3%, indicating in this telomeric region that the distance between the markers is of the order of 0.5 to 1 megabase.

The results indicate that D4S3032*5 and D4S2921*13 show strong association with intermediate phenotypes underlying asthma in two diverse panels of subjects. It may therefore be inferred that a gene influencing asthma is present within 500 kilobases in either direction of D4S3032 and D4S2921.

REFERENCES

1. Strachan, D.P., Anderson, H.R., Limb, E.S., O'Neill, A. and Wells, N. (1994). A national survey of asthma prevalence, severity, and treatment in Great Britain. *Arch. Dis. Child.*, **70**, 174-178.

2. Fraser, R.S., Paré, J.A.P., Fraser, R.G. and Paré, P.D., eds. (1994) Synopsis of Diseases of the Chest. WB Saunders Company, Philadelphia: 635-53.

3. Djukanovic, R., Roche, W.R., Wilson, J.W., Beasley, C.R., Twentyman, O.P., Howarth, R.H. and Holgate S.T. (1990) Mucosal inflammation in asthma. *Am. Rev. Respir. Dis.,* **142**, 434-457.

4. Hill, M.R. and Cookson, W.O.C.M. (1996). A new variant of the β subunit of the high-affinity receptor for Immunoglobulin E (FcεRI-β E237G): Associations of measures of atopy and bronchial hyper-responsiveness. *Hu. Mol. Gen.* **5**, 959-962.

5. Broide, D.H., Lotz, M., Cuomo, A.J., Coburn, D.A., Federman, E.C. and Wassserman, S.I. (1992) Cytokines in symptomatic asthma airways. *J. Allergy Clin. Immunol.* ***89**, 958-967.*

6. Moffatt M.P, Cookson WOCM. Tumour Necrosis Factor Haplotypes. and Asthma. Hum Molec Genet 1997; **6**: 551-554.

7. Bailly S, di Giovine FS, Blakemore A.I.F, Duff G.W. Genetic polymorphism of human interleukin-1α. Eur J Immunol 1993; **23**: 1240-1245.

8. Daniels S.E, Bhattacharyya S, James A, Leaves N.I, Young A, Hill M.R, Faux J.A, Ryan G.F, le Söuef P.N, Lathrop G.M, Musk A.W, Cookson W.O.C.M. A genome-wide search for quantitative trait loci underlying asthma. Nature 1996; **383**: 247-250.

9. Dib S. et al. A comprehensive map of the human genome based on 5264 microsatellites. Nature 1996; **380**: 152-154.

CLAIMS

1. A method for diagnosing an individual as being asthmatic, or as having a predisposition to asthma, which method comprises demonstrating in the individual the presence or absence of one or more alleles which are associated with asthma, wherein the one or more alleles are situated at a locus in a region of chromosome 4 of up to 1 megabase in length, which region contains the locus D4S3032 and/or D4S2921.

2. The method according to claim 1, wherein the method comprises the steps of:

   (i) obtaining a suitable tissue sample from the individual;

   (ii) preparing from the tissue sample a nucleic acid sample;

   (iii) analysing the nucleic acid sample for the presence or absence of the allele.

3. The method according to claim 2, wherein prior to analysis, the locus at which the or each allele is situated is amplified.

4. The method according to claim 3, wherein the amplification is by the PCR.

5. The method according to any one of claims 1 to 4, wherein the locus at which the or each allele is situated comprises microsatellite repeats of variable length.

6. The method according to claim 3 or claim 4, wherein the amplification is performed using a pair of primers for each allele, wherein each primer in a pair hybridises under suitably stringent conditions to a region either side of the microsatellite repeats.

7. The method according to any one of claims 1 to 6, wherein the allele for identification is D4S3032*5.

8. The method according to any one of claims 1 to 6, wherein the allele for identification is D4S2921*13.

9. The method according to any one of claims 1 to 6, wherein the alleles for identification are D4S3032*5 and D4S2921*13.

10. The method according to any one of claims 3 to 9, wherein the analysis is carried out by size separation of amplification products.

11. The method according to claim 10, wherein the primers in the pair of primers comprise the oligonucleotide sequences identified by SEQ ID NO: 1 and SEQ ID NO: 2 or substantially similar sequences, for D4S3032*5; or identified by SEQ ID NO: 3 and SEQ ID NO: 4 or substantially similar sequences, for D4S2921*13; or both of the aforementioned pairs of primers for both of the aforementioned alleles.

12. A pair of oligonucleotide primers for amplification of an allele which is associated with asthma, which allele is situated at a locus in a region of chromosome 2 of up to 1 megabase in length, which region contains the locus D4S3032 and/or D4S2921.

13. The pair of oligonucleotide primers according to claim 12, one of which is labeled with a detectable marker.

14. The pair of oligonucleotides according to claim 12 or claim 13, capable of hybridising under suitably stringent conditions to a region either side of a region of microsatellite repeats at D4S3032 or D4S2921.

15. The pair of oligonucleotide primers according to claim 14, comprising the oligonucleotide sequences identified by SEQ ID NO:1 and SEQ ID NO:2 or substantially similar sequences, for D4S3032*5; or the oligonucleotide sequences identified by SEQ ID NO: 3 and SEQ ID NO:4 or substantially similar sequences, for D4S2921*13.

16. An assay kit which comprises the pair of oligonucleotide primers according to any one of claims 12 to 15.

SEQUENCE LISTING

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<120> POLYMORPHISM II

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<151> 1998-03-27

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/00966

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MARONE G: "Asthma: recent advances" TRENDS IMMUNOLOGY TODAY, vol. 19, no. 1, January 1998 (1998-01), pages 1-5, XP004101456 page 1, paragraph 2	1
A	ABRAMSON M ET AL: "The new asthma genetics and its implications for public health" PUBLIC HEALTH REVIEW, vol. 26, no. 2, February 1998 (1998-02), pages 127-144, XP002110731 see abstract and page 137, para 4 to page 138	1-16

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
29 July 1999	10/08/1999

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/00966

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DANIELS S ET AL: "A genome-wide search for quantitative trait loci underlying asthma " NATURE, vol. 383, 1996, pages 247-50, XP002110730 cited in the application the whole document	1-16
A	WO 95 05481 A (ISIS INNOVATION LTD.) 23 February 1995 (1995-02-23) cited in the application the whole document	1-16
A	DIB C ET AL: "A comprehensive genetic map of the human genome based on 5264 microsatellites" NATURE, vol. 380, 14 March 1996 (1996-03-14), pages 152-154, XP002110732	

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/00966

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9505481	A	23-02-1995	NONE	

Form PCT/ISA/210 (patent family annex) (July 1992)

TENT COOPERATION TREAT

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference KP/3282 PCT	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/GB 99/ 00966	International filing date *(day/month/year)* 26/03/1999	(Earliest) Priority Date *(day/month/year)* 27/03/1998
Applicant ISIS INNOVATION LIMITED et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[X] contained in the international application in written form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

POLYMORPHISM II : LINKAGE OF ASTHMA TO A LOCUS ON CHROMOSOME 4

5. With regard to the **abstract**,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. ______

[ ] as suggested by the applicant.

[X] None of the figures.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

INTERNATIONAL SEARCH REPORT

…tional Application No
…/GB 99/00966

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MARONE G: "Asthma: recent advances" TRENDS IMMUNOLOGY TODAY, vol. 19, no. 1, January 1998 (1998-01), pages 1-5, XP004101456 page 1, paragraph 2 ---	1
A	ABRAMSON M ET AL: "The new asthma genetics and its implications for public health" PUBLIC HEALTH REVIEW, vol. 26, no. 2, February 1998 (1998-02), pages 127-144, XP002110731 see abstract and page 137, para 4 to page 138 ---	1-16
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Date of the actual completion of the international search	Date of mailing of the international search report
29 July 1999	10/08/1999

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DANIELS S ET AL: "A genome-wide search for quantitative trait loci underlying asthma " NATURE, vol. 383, 1996, pages 247-50, XP002110730 cited in the application the whole document ---	1-16
A	WO 95 05481 A (ISIS INNOVATION LTD.) 23 February 1995 (1995-02-23) cited in the application the whole document ---	1-16
A	DIB C ET AL: "A comprehensive genetic map of the human genome based on 5264 microsatellites" NATURE, vol. 380, 14 March 1996 (1996-03-14), pages 152-154, XP002110732 -----	

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INTERNATIONAL SEARCH REPORT

[redacted]n on patent family members

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Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9505481	A	23-02-1995	NONE	

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(54) Title: POLYMORPHISM II: LINKAGE OF ASTHMA TO A LOCUS ON CHROMOSOME 4

(57) Abstract

A method for diagnosing an individual as being asthmatic, or as having a predisposition to asthma is described, which method comprises demonstrating in the individual the presence or absence of one or more alleles which are associated with asthma, wherein the one or more alleles are situated at a locus in a region of chromosome 4 of up to 1 megabase in length, which region contains the locus D4S3032 and/or D4S2921.

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POLYMORPHISM II: LINKAGE OF ASTHMA TO A LOCUS ON CHROMOSOME 4

This invention is concerned with methods for the diagnosis of asthma and with materials and methods relating thereto.

Asthma is a disease which is becoming more prevalent and is the most common disease of childhood (1). Most asthma in children and young adults is initiated by IgE mediated allergy (atopy) to inhaled allergens such as house dust mite and cat dander. However, not all asthmatics are atopic, and most atopic individuals do not have asthma, so that factors in addition to atopy are necessary to induce the disease (2,3). Asthma is strongly familial, and is due to the interaction between genetic and environmental factors. The genetic factors are thought to be variants of normal genes ("polymorphisms") which alter their function to predispose to asthma.

Asthma may be identified by recurrent wheeze and intermittent air flow limitation. An asthmatic tendency may be quantified by the measurement of bronchial hyper-responsiveness in which an individual's dose-response curve to a broncho-constrictor such as histamine or methacholine is constructed. The curve is commonly summarised by the dose which results in a 20% fall in air flow (PD20) or the slope of the curve between the initial air flow measurement and the last dose given (slope). Asthma is accompanied by blood eosinophilia, and eosinophils are prominent in asthmatic airways.

In the atopic response, IgE is produced by B-cells in response to allergen stimulation. These antibodies coat mast cells by binding to the high affinity receptor for IgE (FcεRI). When a multivalent allergen binds to an IgE-coated mast cell, the cross-linking of adjacent IgEs by allergen initiates a series of cellular events leading to the destabilisation of the cell membrane

and release of inflammatory mediators. This results in mucosal inflammation, wheezing, coughing, sneezing and nasal blockage.

Atopy can be diagnosed by (i) a positive skin prick test in response to a common allergen; (ii) detecting the presence of specific serum IgE for allergen; or (iii) by detecting elevation of total serum IgE.

Genetic factors underlying a disease may be identified through localisation to particular chromosomal regions by genetic linkage. Genetic linkage is established by the study of families. It relies on matching the inheritance of disease with genetic polymorphisms of known localisation (known as "genetic markers"). In a complex disease such as asthma, genetic linkage will typically localise genes to within 10 - 20 Megabases (Mb) of DNA. A region of this size may contain 350 - 700 genes, and will be too large to permit immediate identification of the disease-causing gene.

Closer localisation of disease-causing genes may be accomplished by the detection of associations between particular alleles and the disease phenotype. Over short segments of DNA, distinctive alleles of the individual polymorphisms will show non-random association with alleles of neighbouring polymorphisms. This phenomenon, known as "linkage disequilibrium" occurs over 50-500 Kilobases (Kb) of DNA. Linkage disequilibrium may be detected by the study of individuals as well as by the study of families.

Disease-causing alleles will be in linkage disequilibrium with non-functional polymorphisms from the same chromosomal segment. It is therefore possible to detect allelic association with disease from particular chromosomal segments, without identifying the exact polymorphism and gene underlying the disease state.

The detection of allelic association may therefore give information as to disease susceptibility in a particular individual. Furthermore,

allelic association is indicative of a disease-causing gene being present within 500 Kb of DNA in either direction from the allele (i.e. 1 Mb in total). Such a region may contain only 30 genes, within which the identification of the disease-causing gene is possible.

The presence of linkage disequilibrium also means that other polymorphisms may be anticipated to associate with disease, and that these additional polymorphisms will also be diagnostic of disease susceptibility in particular individuals.

Genetic associations with atopy have been demonstrated. WO 95/05481 discloses that variants of the gene encoding the β-subunit of the high-affinity receptor for IgE (FcεRIβ) are associated with atopy. It teaches a method for diagnosing atopy which is based upon the demonstration of the presence or absence of one of two variants in a specific portion of the DNA sequence of the gene encoding FcεRIβ, located near the commencement of exon 6 of the FcεRIβ gene on chromosome 11. A further variant has also been found in which the unusual variant sequence is in the coding sequence for the C-terminal cytoplasmic tail of FcεRIβ (4).

Tumour Necrosis Factor (TNF) is a pro-inflammatory cytokine that is found in increased concentration in asthmatic airways (5). We have previously shown that polymorphisms within the TNF gene are associated with an increased risk of asthma (6).

The known polymorphisms do not account for all of the genetic factors which predispose to asthma. In particular, asthma is not necessarily an atopic disease. Identification of further genetic polymorphisms linked to asthma will allow the identification of children at risk of asthma before the disease has developed (for example immediately after birth), with the potential for prevention of disease. The presence of particular polymorphisms may predict the clinical course of disease (e.g. severe as opposed to mild) or the

response to particular treatments. This diagnostic information will be of use to the health care, pharmaceutical and insurance industries.

We have previously established linkage of bronchial hyper-responsiveness to chromosome 4 (8). However, this finding is of no use in diagnosis.

It has now been discovered that a genetic polymorphism known as D4S3032*5 on chromosome 4 and a nearby polymorphism known as D4S2921*13 are associated with asthmatic traits. Specifically, D4S3032*5 is associated with bronchial hyper-responsiveness and D4S2921*13 is associated with peripheral eosinophilia, both of these being traits which underlie asthma. The two polymorphisms can therefore be used as diagnostic tools.

The invention therefore provides a method for diagnosing an individual as being asthmatic, or as having a predisposition to asthma, which method comprises demonstrating in the individual the presence or absence of one or more alleles which are associated with asthma, wherein the one or more alleles are situated at a locus in a region of chromosome 4 of up to 1 megabase in length, which region contains the locus D4S3032 and/or D4S2921.

The 1Mb region of chromosome 4 referred to flanks the D4S3032 and D4S2921 loci. Thus, the specific allele D4S3032*5, or D4S2921*13, or other unusual polymorphisms in the region which are associated with asthma, may be the subject of identification in the method according to the invention. Equally two or more such alleles may be the subject of identification, including in particular the combination of D4S3032*5 and D4S2921*13.

Current diagnostic methods involving detection at the nucleic acid level normally comprise the steps of:

(i) obtaining a suitable tissue sample from the individual;
(ii) preparing from the tissue sample a nucleic acid sample;
(iii) analysing the nucleic acid sample for the presence or absence of the relevant nucleic acid sequence, such as a specific allele.

Preferably, an amplification step is performed prior to the analysis, such that the locus at which the allele is situated is amplified. A preferred amplification technique is the PCR, although any suitable method of nucleic acid amplification may be employed.

In further aspects, the invention provides a pair of oligonucleotide primers for amplification of an allele which is associated with asthma, which allele is situated at a locus in a region of chromosome 4 of up to 1 megabase in length, which region contains the locus D4S3032 and/or D4S2921; and an assay kit comprising the pair of oligonucleotide primers.

The specific allele for identification may take the form of microsatellite repeats, which are nucleotide sequences containing short, repeated nucleotide motifs, usually a dinucleotide or a trinucleotide motif. A pair of primers which hybridize under suitably stringent conditions, to sequences at a position on either side of the microsatellite repeats, may be used to amplify the microsatellite repeats by PCR. Differences in the number of repeats are recognised by size differences in the PCR products. An allele which has a specified number of repeats and therefore a known size can thus be identified. D4S3032*5 and D4S2921*13 are examples of such alleles.

The primers employed in the method comprise nucleic acid sequences which are complementary to, or substantially complementary to unique sequences either side of the microsatellite repeats, such that only the relevant polymorphic region of the genome is amplified. The conditions under which the amplification is performed are gauged such that specific

hybridization of the primers to the flanking sequences occurs and non-specific hybridization is avoided. The hybridization conditions are suitably stringent for that purpose. Standard techniques can be used to identify an appropriate set of reaction conditions.

Typically, the PCR products are detected by means of a detectable label attached to one of the PCR primers. Alternatively another form of labeling may be used such as a labeled sequence specific probe which hybridizes to the amplified sequences. The label may be a fluorescent or other label. The PCR products are subjected to size determination, typically involving size-separation for example by gel electrophoresis, and the presence or absence of the allele of interest is determined.

It will be evident that the invention is not limited with regard to the manner in which the presence or absence of the allele of interest is determined. The labeling, detection, separation or any other aspect of the method as described here may be replaced by other suitable known techniques and reagents.

The allele for identification may be an allele other than D4S3032*5 or D4S2921*13 which is in linkage disequilibrium with D4S3032*5 or D4S2921*13 and is associated with asthma. This includes alleles of both functional and non-functional polymorphisms. Functional polymorphisms include polymorphisms within genes, usually within coding sequences of genes. Non-functional polymorphisms are polymorphisms which do not themselves cause the disease.

This invention will now be further described in the Examples section which follows. The Examples are intended to be illustrative and do not limit the scope of the invention in any way.

EXAMPLES

Description of Laboratory Testing

Subjects

Two panels of subjects have been studied.

Panel A consisted of 80 nuclear families sub-selected from an Australian population sample of 230 families (8). The panel contained a total of 203 offspring forming 172 sib-pairs. 12% of the children were asthmatic.

Panel C consisted of 87 nuclear families recruited through a child attending an asthma clinic in the Oxford region. The families contained 216 offspring (148 sibling pairs), of whom 44% were asthmatic.

Phenotyping

Bronchial responsiveness to methacholine was measured as previously described (8): the maximum dose administered was 12 μmol. The slope of the dose-response curve was calculated as (pre-dose forced expiratory volume in one second (FEV1) - last FEV1) ÷ the cumulative dose of methacholine). A constant of 0.01 was added to each measurement, to allow loge transformation when Slope was ≤ 0. Eosinophils in peripheral blood were Coulter-counted and the values $\log_e$ transformed before analysis.

Genotyping

The microsatellite markers D4S3032 and D4S2921 were typed by semi-automated fluorescent methods, as described previously (8). These markers are in close proximity at the telomeric region of the long arm of chromosome 4.

The polymerase chain reaction primer sequences for the markers were as follows:

D4S3032	5' TGA AAT TCT ATT GAC CAA TGA TGT G (SEQ ID NO: 1)
UD4S3032	5' TAG CAC CTG GAT TTA CCA TGA C (SEQ ID NO: 2)
D4S2921	5' TCC TTC AGG AAC TGG TG (SEQ ID NO: 3)
UD4S2921	5' TTA AAA ATC TAC AGA CAA GGG C (SEQ ID NO: 4)

The polymerase chain reaction conditions were as follows: The reaction volumes were 10μl, containing 50ng of genomic DNA, 200mM dNTPs, 1 x NH4+ buffer, 50ng oligonucleotide primers (forward labelled fluorescently), 0.5 to 3.0mM $MgCl_2$ and 0.2U Taq polymerase. Cycling conditions were 1 min at 95°C, 1 min at 55°C and 45s at 72°C; 28 cycles were used. PCRs were performed on an Hybaid Omnigene thermal cycler.

Electrophoresis and allele scoring were as follows:

PCR products were mlxed with a size standard (GS350 TAM) in loading buffer (80% (v/v) formamide, 20% (vlv) 50mM EDTA, 0.1% (w/v) blue dextran). Samples were denatured at 95°C for 4 min immediately prior to loading onto a 6% polyacrylamide gel and were electrophoresed at 800v for 6h on an Applied Biosystems (ABI) 313 DNA sequencer. Allele sizes were assigned using the ABI GENESCAN and ABI GENOTYPER software.

Association Analysis

Association was tested against the phenotype of bronchial hyper-responsiveness and peripheral blood eosinophil counts by the ASSOC routine of the SAGE (ver2.2) computer program.

Results

Association with D4S3032 allele 5 and D4S2921 allele 13

Each of the markers was then tested for association with the asthma phenotype. Association was seen in panel A for allele 5 of D4S3032

(D4S3032*5) and bronchial hyper-responsiveness. This allele is 145 base pairs in size, using the primers described above. Association between allele 13 of D4S2921 (D4S2921*13) and eosinophil counts were seen in both panels. This allele is 162 base pairs in size, using the primers described above. (Other suitable primers can be designed and their amplification product size determined for D4S3032*5 or D4S2921*13, using known sequence information (9).) The results of testing were as follows:

			Panel A		Panel C		Combined	
Trait	Marker	Allele	χ^2	p	χ^2	p	χ^2	p
Slope	d4s3032	5	13.56	0.0002	-	-	-	-
Eosinophils	d4s2921	13	5.00	0.03	11.17	0.0008	17.61	0.0000

The recombination fraction between D4S3032 and D4S2921 was 3%, indicating in this telomeric region that the distance between the markers is of the order of 0.5 to 1 megabase.

The results indicate that D4S3032*5 and D4S2921*13 show strong association with intermediate phenotypes underlying asthma in two diverse panels of subjects. It may therefore be inferred that a gene influencing asthma is present within 500 kilobases in either direction of D4S3032 and D4S2921.

REFERENCES

1. Strachan, D.P., Anderson, H.R., Limb, E.S., O'Neill, A. and Wells, N. (1994). A national survey of asthma prevalence, severity, and treatment in Great Britain. *Arch. Dis. Child.*, **70**, 174-178.

2. Fraser, R.S., Paré, J.A.P., Fraser, R.G. and Paré, P.D., eds. (1994) Synopsis of Diseases of the Chest. WB Saunders Company, Philadelphia: 635-53.

3. Djukanovic, R., Roche, W.R., Wilson, J.W., Beasley, C.R., Twentyman, O.P., Howarth, R.H. and Holgate S.T. (1990) Mucosal inflammation in asthma. *Am. Rev. Respir. Dis.,* **142**, 434-457.

4. Hill, M.R. and Cookson, W.O.C.M. (1996). A new variant of the β subunit of the high-affinity receptor for Immunoglobulin E (FcεRI-β E237G): Associations of measures of atopy and bronchial hyper-responsiveness. *Hu. Mol. Gen.* **5**, 959-962.

5. Broide, D.H., Lotz, M., Cuomo, A.J., Coburn, D.A., Federman, E.C. and Wassserman, S.I. (1992) Cytokines in symptomatic asthma airways. *J. Allergy Clin. Immunol.* ***89**, 958-967.*

6. Moffatt M.P, Cookson WOCM. Tumour Necrosis Factor Haplotypes. and Asthma. Hum Molec Genet 1997; **6**: 551-554.

7. Bailly S, di Giovine FS, Blakemore A.I.F, Duff G.W. Genetic polymorphism of human interleukin-1α. Eur J Immunol 1993; **23**: 1240-1245.

8. Daniels S.E, Bhattacharyya S, James A, Leaves N.I, Young A, Hill M.R, Faux J.A, Ryan G.F, le Söuef P.N, Lathrop G.M, Musk A.W, Cookson W.O.C.M. A genome-wide search for quantitative trait loci underlying asthma. Nature 1996; **383**: 247-250.

9. Dib S. et al. A comprehensive map of the human genome based on 5264 microsatellites. Nature 1996; **380**: 152-154.

CLAIMS

1. A method for diagnosing an individual as being asthmatic, or as having a predisposition to asthma, which method comprises demonstrating in the individual the presence or absence of one or more alleles which are associated with asthma, wherein the one or more alleles are situated at a locus in a region of chromosome 4 of up to 1 megabase in length, which region contains the locus D4S3032 and/or D4S2921.

2. The method according to claim 1, wherein the method comprises the steps of:

    (i) obtaining a suitable tissue sample from the individual;

    (ii) preparing from the tissue sample a nucleic acid sample;

    (iii) analysing the nucleic acid sample for the presence or absence of the allele.

3. The method according to claim 2, wherein prior to analysis, the locus at which the or each allele is situated is amplified.

4. The method according to claim 3, wherein the amplification is by the PCR.

5. The method according to any one of claims 1 to 4, wherein the locus at which the or each allele is situated comprises microsatellite repeats of variable length.

6. The method according to claim 3 or claim 4, wherein the amplification is performed using a pair of primers for each allele, wherein each primer in a pair hybridises under suitably stringent conditions to a region either side of the microsatellite repeats.

7. The method according to any one of claims 1 to 6, wherein the allele for identification is D4S3032*5.

8. The method according to any one of claims 1 to 6, wherein the allele for identification is D4S2921*13.

9. The method according to any one of claims 1 to 6, wherein the alleles for identification are D4S3032*5 and D4S2921*13.

10. The method according to any one of claims 3 to 9, wherein the analysis is carried out by size separation of amplification products.

11. The method according to claim 10, wherein the primers in the pair of primers comprise the oligonucleotide sequences identified by SEQ ID NO: 1 and SEQ ID NO: 2 or substantially similar sequences, for D4S3032*5; or identified by SEQ ID NO: 3 and SEQ ID NO: 4 or substantially similar sequences, for D4S2921*13; or both of the aforementioned pairs of primers for both of the aforementioned alleles.

12. A pair of oligonucleotide primers for amplification of an allele which is associated with asthma, which allele is situated at a locus in a region of chromosome 2 of up to 1 megabase in length, which region contains the locus D4S3032 and/or D4S2921.

13. The pair of oligonucleotide primers according to claim 12, one of which is labeled with a detectable marker.

14. The pair of oligonucleotides according to claim 12 or claim 13, capable of hybridising under suitably stringent conditions to a region either side of a region of microsatellite repeats at D4S3032 or D4S2921.

15. The pair of oligonucleotide primers according to claim 14, comprising the oligonucleotide sequences identified by SEQ ID NO:1 and SEQ ID NO:2 or substantially similar sequences, for D4S3032*5; or the oligonucleotide sequences identified by SEQ ID NO: 3 and SEQ ID NO:4 or substantially similar sequences, for D4S2921*13.

16. An assay kit which comprises the pair of oligonucleotide primers according to any one of claims 12 to 15.

SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

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B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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A	ABRAMSON M ET AL: "The new asthma genetics and its implications for public health" PUBLIC HEALTH REVIEW, vol. 26, no. 2, February 1998 (1998-02), pages 127-144, XP002110731 see abstract and page 137, para 4 to page 138 --- -/--	1-16

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INTERNATIONAL SEARCH REPORT

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International Application No
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Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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(57) Abstract

A method of diagnosing atopy or a predisposition to atopy in an individual, which comprises demonstrating the presence of a mutation or polymorphism in a specific DNA sequence of a gene encoding the beta-subunit of the high affinity IgE receptor in the individual. Two variant DNA sequences linked with atopy are as follows: 5' GAA TTG GTA TTG ATG (SEQ ID NO: 2), 5' GAA TTG GTA GTG ATG (SEQ ID NO: 4), both commencing at nucleotide 5640 of the beta-subunit gene. The invention makes it possible for the first time to identify individuals at genetic risk of developing atopic illness.

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DIAGNOSTIC METHOD AND THERAPY

The invention relates to diagnosis of atopy or of a predisposition to atopy, and to treatment of atopic or potentially atopic individuals.

Atopy is a heterogeneous disorder characterised by prolonged and enhanced immunoglobulin E(IgE) responses to common environmental antigens, including pollens and house dust mites; it underlies the common diseases of allergic asthma and rhinitis (hay fever). The high-affinity receptor for IgE (FcϵRI) binds IgE to mucosal mast cells and plays a central role in allergy (1). When allergen binds to mast cell bound IgE, FcϵRI initiates a series of events leading to the cellular release of inflammatory mediators. This results in mucosal inflammation and the characteristic symptoms of wheezing, coughing, sneezing and nasal blockage.

Atopy may be detected by positive skin prick tests of common allergens, by the presence of specific serum IgE against these allergens or by elevation of the total serum IgE. These three variables are strongly correlated with each other and with the presence of symptoms. Atopy, when defined as a prick skin test response to one or more common allergens, affects up to 50% of Western populations. As a result of atopy, as many as 10% of children suffer from asthma. Atopy results from complex interactions between heterogeneous genetic and environmental factors. The factors that govern the development of generalized atopic responsiveness, a characteristic of most atopics as they respond to many allergens, probably differ from those determining allergic

response to any particular allergen or specific allergic symptoms.

Using quantitative assays for IgE response to allergens, we have observed genetic linkage between generalized atopic IgE responses and chromosome 11q in a data set which includes over 300 affected sibling-pairs (2-6). This linkage is robust to phenotype classification (6). The data suggest that 60% of families, when ascertained through a young symptomatic atopic proband, are linked to chromosome 11q (5). Notably, the sharing of alleles from chromosome 11 by atopic sibling-pairs is exclusively from maternal chromosomes (4). This observation accords with data from large epidemiological studies suggesting a maternal transmission of atopy (7-9). It is consistent with a maternal effect on fetal or neonatal immune development or with paternal genomic imprinting. The interactions of the 11q locus with other genetic loci and environmental factors in determining the atopic disease phenotype remain to be determined.Early attempts at independent replication of linkage to chromosome 11q, however, have produced variable results. Genetic heterogeneity and methodological factors, in particular the numbers of families and individuals tested, account for the discrepancies. Four studies have reported negative linkage (10-13), but two contained insufficient information to confirm or exclude linkage of atopy to the marker D11S97 on chromosome 11 (10,11). Inspection of the raw data from a third study (12) of three extended pedigrees shows a maximum lod score of 1.7 at 0 recombination in one family; the other two families show paternal inheritance and non-linkage of atopy. The fourth study, of mixed extended and nuclear families, tested linkage with the locus Int2 which is telomeric to D11S97, although atopy had previously been reported as

10% centromeric to the marker; the lod score was-2 at 10% recombination (13). In addition, none of these studies took account of the maternal linkage to chromosome 11. In contrast, data from Japan, using lod scores (14), and the Netherlands, using affected sib-pair methods (15), have confirmed linkage in families with marked symptomatic atopy. Because the atopy is a complex genetic disease, we believe that genetic linkage is more satisfactorily demonstrated and analysed using affected sibling-pair methods; these are not dependent upon an assumed mode of inheritance and control for penetrance and environmental effects (4).

In linkage mapping of atopy on chromosome 11q we have defined a confidence interval for the localisation of the atopy locus around 2 homologous genes, CD20 and the β-subunit of FcεRI (5). CD20 is a proliferation and differentiation factor in B-lymphocyte lineage whose function is not known to be related to atopic IgE responses. We have previously found that CD20 Msp1 restriction alleles (16) are not associated with atopy in children from unrelated nuclear families (odds ratio for alleles A and B = 0.95, 95%CI 0.56-1.60) (5).

The Invention

We have now established that variants of the gene encoding the beta-subunit of the high-affinity receptor for IgE are associated with atopy. Surprising results have revealed that mutations or variants in the gene alter the risk of an individual being atopic. This finding makes possible for the first time the strategy of diagnosis.

The present invention provides a method of diagnosing atopy or a predisposition to atopy in an individual, which method comprises demonstrating the presence of a mutation or polymorphism in a specific DNA sequence of a gene encoding the beta-subunit of the

high affinity IgE receptor in the individual.

In a particular embodiment, the gene is on chromosome 11q. More particularly, the specific DNA sequence is located near the commencement of exon 6 of the gene on chromosome 11q.

Gene variants have been found near the commencement of exon 6 on chromosome 11q. This exon runs from nucleotide 5640 to 5738 of the beta-subunit gene. The wild type (normal) sequence at this site, commencing with nucleotide 5640 is:

5´ GAA ATT GTA GTG ATG (SEQ ID NO: 1)

The full normal sequence of the beta-subunit gene has been published (17) and can be found in the Genbank and Embl Databases, Accesssion No. M89796.

Two variant sequences have now been identified. The first, commencing at nucleotide 5640 is:

(i) 5´ GAA TTG GTA TTG ATG (SEQ ID NO: 2)

This results in a substitution of the amino acid leucine for isoleucine at position 181 and substitution of leucine for valine at position 183.

The second variant, commencing at nucleotide 5640, is:

(ii) 5´ GAA TTG GTA GTG ATG (SEQ ID NO: 4)

This results only in substitution of leucine for isoleucine at position 181.

In the method of diagnosis according to the invention, the specific DNA sequence may thus comprise one of the above sequences (i) and (ii), or a relevant portion thereof. A relevant portion is a portion which

is different to the wild type sequence.

The method may comprise amplification of the specific DNA sequence or a relevant portion thereof.

One amplification technique which may be used is the amplification refractory mutation system (ARMS) PCR technique. Another is PCR, which may be followed by probing of the amplification products with a sequence-specific nucleic acid probe capable of annealing to a relevant portion of the amplified specific DNA sequence. Other DNA or RNA-based methods may also be used.

In the ARMS technique, at least one primer is used which anneals to a DNA sequence comprising the mutant or variant sequence, but not to the wild type sequence. Thus, only when the mutation or polymorphism is present will there be successful PCR amplification. Further confirmation may be obtained by probing or sequencing or by other known methods.

Suitable primers for amplification of sequences in exon 6 of the beta-subunit gene can be devised from the known DNA sequence, and in the case of ARMS, from the variant sequences (i) and (ii) above.

The method of diagnosis according to the invention may thus be performed on a DNA sample, but the invention is not limited to testing DNA. The method may instead be performed on a product of the specific DNA sequence, such as messenger RNA (mRNA). Or the mutation or polymorphism may be identified in cDNA made from mRNA.

Alternatively, the method may involve identifying the presence of a variant peptide or protein derived from the specific DNA sequence. For instance, antibodies raised against the variant peptide sequence may be labelled and used for in vitro or in vivo diagnosis. The variant peptide sequence can be synthesised by standard techniques eg. using an

automatic synthesiser. The antibodies can be made by administering the peptide in antigenic form to a suitable host. Polyclonal or monoclonal antibodies may be prepared by standard techniques.

The invention provides peptides corresponding to variants of exon 6 of the gene encoding the high affinity IgE receptor on chromosome 11q, and phosphorylation and glycosylation products, and characteristic fragments thereof.

Such a peptide preferably comprises the amino acid sequence:

Glu Leu Val Leu Met (SEQ ID NO: 3) or
Glu Leu Val Val Met (SEQ ID NO: 5),

or a relevant portion thereof. A relevant portion is a portion which is different to the wild type. The two above-mentioned amino acid sequences correspond to the variant nucleic acid sequences (i) and (ii).

The invention also provides antibodies to the variant peptides described above, and fragments of the antibodies. the antibodies or fragments will be useful in the method of diagnosis according to the invention, to identify protein variants.

In another aspect, the invention provides, as new chemical compounds, nucleic acids comprising the sequence (i) or (ii) above or complementary DNA or RNA.

In a particular emdodiment, the invention provides a nucleic acid comprising a first portion which corresponds substantially to the whole or part of exon 6 of the gene encoding the beta-subunit of the high-affinity receptor for IgE, which first portion includes one of the following sequences:

5´ TTG GTA TTG or
5´ A TTG GTA GTG (SEQ ID NO: 6) or
5´ TTG GTA GTG A (SEQ ID NO: 7)

or complementary DNA or RNA, and optionally a second portion which corresponds substantially to the whole or part of an intron adjacent to said exon or complementary DNA or RNA.

Probes comprising a wild type or variant oligonucleotide or a nucleic acid as described herein, linked to a signal moiety or immobilised on a surface, are also considered to be part of the invention. Variant probes will be useful for identifying variant phenotypes and wild type probes can be used for control purposes.

Detailed Description

The invention therefore provides diagnostic tests for functional polymorphisms within and close to the beta chain gene. These tests may be used for post-natal diagnosis of an atopic predisposition, in order to carry out preventative measures against allergen sensitisation in early childhood. The tests may also identify asthmatic or other atopic subjects who respond to particular treatment modalities. The tests may also identify individuals susceptible to industrial asthma, or to the effects of cigarette smoke and other pollutants.

The recognition that the beta chain predisposes to asthma permits novel methods of treatment of asthma (and other atopic illnesses such as allergic rhinitis and eczema) directed at the beta chain, such as pharmacologic blocking of its action. The invention also provides treatments arising from recognition that variation in the beta chain is central to the atopic state, and methods for developing such treatments. Treatments may be developed for example by testing pharmacologic compounds against cell systems (eg. monkey cos cells) containing the receptor genes. Effects of pharmacologic compounds can be tested on

wild type and variant-encoded receptors, to look for compounds which eg. down-regulate the variant receptor but not the wild type receptor. High throughput screening assays will be possible. In other words, the mutant beta chain would be part of an assay to develop new drugs, or proteins to alter the receptor function . A strategy based on "antisense RNA" to block the action of the beta chain can also be envisaged.

The mutations discussed above were found in atopic individuals and their families. Initially genomic DNA was sequenced from each of the seven exons and splice sites of <u>FcεRI-β</u> in six atopic and six non-atopic individuals. One atopic individual was found to have a chromosome with three nucleotide substitutions in the 6th exon, resulting in Ile181Leu and Val183Leu substitutions within the 4th transmembrane domain (TM) of FcεRI-β (17) (Fig. 1). Details are given in Example 1.

The prevalence of leucine residues at positions 181 and 183 of FcεRI-β and their relationship to atopy were defined using allele specific DNA amplification (ARMS) (18), as described in Example 2. In a random patient sample, Leu181 shows association with atopy. But in accordance with the documented maternal inheritance of atopy on chromosome 11q, 11 of 24 (46%) Leu181 heterozygotes in the random patient sample were non-atopic.

Family studies were carried out to clarify the relationship between genotype and phenotype (Example 2). In each of 10 atopic families in which Leu181 was found, transmission was through the mother and a strong association between the variant and atopy was demonstrable in the children.

The strong association between maternally inherited Leu181 and atopy in a set of unrelated families indicates variants of FcεRI-β as one cause of

atopic IgE responsiveness. This is consistent with the known biological functions of the high affinity IgE receptor (1,19). FcϵRI is comprised of three subunits α, β and $gamma_2$; in human, α and gamma are encoded on chromosome 1 and the β subunit on chromosome 11 (5). FcϵRI is expressed on mast cells, basophils, monocytes and Langerhans´cells. The receptor plays a central role in the mediation of IgE dependent allergic inflammation (1) but also in IgE metabolism and mast cell and B-lymphocyte differentiation and growth. Stimulation of FcϵRI causes release from the mast cell of cytokines, including IL-4, which are implicated in the up-regulation of mast cell and helper T-cell subtype 2 (TH2) development and of IgE production by B-lymphocytes. Lung mast cells that express cell contact signals including CD40 ligand may, in the presence of IL-4, regulate local B lymphocyte IgE production independently of T lymphocytes. Variants of FcϵRI-β might promote the atopic state either by enhanced release of pro-inflammatory mediators by mast cells (to cause more symptomatic disease) or by enhanced mast cell expression of IL-4 and CD40 ligand (to cause more local B lymphocyte IgE production).

In the atopic subject originally found to possess Leu181 and Leu183 variants, no other mutation was detected in full coding and splice site sequences of FcϵRI-β. Alpha helical TM domains play an important part in the function of FcϵRI and similar receptors in which non-ionic interactions between non-polar amino acids regulate the relationship of the helices and influence signal transduction. Mutagenicity studies on the FcϵRI subunits show substituting amino acids in TM domains can cause significant changes in the receptor´s expression and function (20). Single amino acid changes within TM domains of other seven-helix bundle receptors have major functional effects; these include

10-20-fold changes in ligand binding in the 5-hydroxytryptamine receptor (26). The exchanges of aliphatic amino acids (Ise-Val-Leu) within a TM of FcϵRI-β parallel species-specific variants of the brain cholecystokinin-B/gastrin receptor which result in 20-fold altered affinity for benzodiazepine-based antagonists (29). It may be significant that substitution of leucines at positions 181 and 183 in human FcϵRI-β generates the same sequence documented in rodents (21,22).

Our observations that 60% of families with an atopic asthmatic are maternally linked to chromosome 11 and that Leu181 occurs in 17% suggest that other variants or mutations of FcϵRI-β are to be expected.

An investigation was carried out on 1004 individuals in 232 two-generation families from an Australian population (Example 3). Within this population sample, maternal inheritance of FcϵRI-β Leu181/Leu183 is strongly associated with atopic IgE responses, elevated eosinophil counts, and bronchial hyper-responsiveness. Children with the variant had greater skin prick tests and RASTs to HDM than other atopic children. The variant therefore identifies a genetic risk factor for marked atopy. A 4.5% prevalencein this population implies that Leu181/Leu183 should be considered to be a polymorphism or variant of normal, rather than a mutation.

It is of note that the "Irish" variant Leu181/Leu183 was found exclusively in the Australian population, although Leu181 seems much more common in English subjects (Examples 1 and 2). This indicates possible variation between populations.

The results make it clear that, in order to interpret the presence of Leu181 or Leu181/Leu183, the maternal or paternal origin of the allele needs to be known. In the Australian study, the completely

negative skin tests and specific IgE titres of subjects who have inherited Leu181/Leu183 paternally was unexpected, given the high background level of atopy. Possible mechanisms for the maternal effect include genomic imprinting or maternal influences through the placenta or breast milk (4). A significant and opposite paternal effect, if confirmed, would favour genomic imprinting as a cause of these phenomena.

One aim of defining the genetic components of atopy has been the identification of individuals at genetic risk of developing atopic illnesses. The present results indicate that polymorphism in FcεRI-β is one factor that can be used to assign such risk. As the timing and degree of exposure to allergen in early life may determine subsequent probability of atopic disease (27), recognition of genetic susceptibility and manipulation of the environment in these individuals may result in effective prevention of illness and morbidity (28).

Reference is directed to the accompanying drawings, in which:-

Figure 1 is a schematic model of the β-subunit of FcεRI(3) demonstrating four transmembrane domains and the position of the leucine substitutions (181 and 183 as solid symbols) within the 4th transmembrane domain, and

Figure 2 shows results of ARMS testing for Leu181 in 60 nuclear families identified through an asthmatic proband. The 10 families with the variant are shown. No family was found with Leu183 variant.

EXAMPLES

Example 1

Six atopic and 6 non atopic individuals were selected for initial DNA sequence analysis.

Atopy phenotype testing.

Atopy was defined as described (30,31), by the presence of a total serum IgE elevated above normal values (Phazedym PRIST, Pharmacia), or a positive skin prick test to house dust mite or grass pollen allergens (Dome-Hollister-Stier, Spokane, USA) ≥ 2mm > a negative control, or a positive specific IgE titre > 0.35 KU_AL^{-1} for the same allergens (Pharmacia CAP system). Individuals with raised total IgE alone but who were smokers were designated as unknown phenotype.

DNA sequence analysis.

DNA sequence spanning all 7 exons and their splice donor and acceptor sites of FcεRI-β was generated by PCR from genomic DNA of 6 atopic and 6 non-atopic individuals. The reaction mixture contained 1μg of genomic DNA in a buffer ($MgCl_2$ 1.5mmol L^{-1} Tris 100 mmol L^{-1}, KCl 500 mmol L^{-1}, gelatin 1mg ml^{-1}), with 200 μM of dNTPs, 0.5μl *Taq* polymerase, and 10% DMSO made up to a final volume of 100 μL. The primers for exons 1 to 3 (reaction 1) were: 5´-TGG GGA CAA TTC CAG AAG AAG-3´ and 5´ - CCG GAA TTC AGG TTT CTC ATG GGA TAA - 3´; and for exons 4 to 6 (reaction 2) were : 5´-TTA GGT GTC TCT CAA CCC ATC-3´ and 5´-CCG GAA TTC CTC ACA AGC CTT CTG TAC-3´; and for exon 7 (reaction 3) were: 5´-CAG CTA ACT TAG GAG GCT GAG-3´ and 5´-TAT CAG GCG AAT AAA TCT AAT GTA-3´. 25 cycles of PCR were carried out for each reaction. The products were then cut with restriction enzymes: reaction 1 used *Bam*HI, *Pst*I and *Eco*RI to give two major fragments of 0.7 and 1.7kb. The product of reaction 2 was digested with *Sma*I and *Eco*RI to yield one major fragment of 2.4 kb; reaction 3 was digested with *Sma*I and *Bam*HI to give a single major fragment of 0.7 kb. The four fragments were cloned into M13 by standard methods. After checking inserts with a forward universal primer, single-strand

sequencing was carried out by the dideoxy chain termination method with the following exon-specific primers: exon 1, 5´-GTT TTC CCA GTC ACG ACG T*-3´; exon 2, 5´-GGT CAG TTA CTT GGA TGC TC-3´; exon 3, 5´-ACA GTC TAG GAC ACT AAC GC-3´; exon 4, 5´-GGA TTA CAG ACA TGA GCC AC-3´; exon 5, 5´-AGA CCG TAC GTG TTC ATG TG-3´; exon 6, 5´-GTC AGA TGG TAG GGA GAT G-3´;exon 7, 5´-GTT TTC CCA GTC ACG ACG-T*-3´ (*indicates M13 - 40 forward primer). Six clones were sequenced for each exon from each individual. Mutations were considered to be present if seen in 2 or more clones.

One atopic individual was found to have a chromosome with three nucleotide substitutions in the 6th exon, resulting in Ile181Leu and Val183Leu substitutions within the 4th TM domain of FcεRI-β, as discussed above.

Example 2

For association studies between FcεRI-β variants and atopy, two groups were studied: (i) A random patient sample of 163 males and females aged 15 -40 years having blood counts carried out at the John Radcliffe Hospital. (ii) 60 nuclear families freshly recruited through atopic asthmatic probands under the age of 21 attending hospital or general practitioner clinics in Oxfordshire. These families had not previously been assessed for linkage to chromosome 11 markers.

Atopy phenotype testing was carried out as described in Example 1. In the random patient sample, total and allergen-specific serum IgE´s were assayed but skin prick test and clinical data were not available.

Allele specific DNA amplification (ARMS) for Leu181 and Leu183.

The Arms method applied was modified from ref.(18). For FcεRI-β, the primers to give a 237 bp band were: a universal upstream primer 5´-AAG TTA TCT ACT GCA AGT GAC GAT CTC T-3´ (SEQ ID NO: 8) together with downstream primers to detect: wild type sequence (Ile181, Val183), 5´-GGT GAG AAA CAG CAT CAT CAC TAC AAT-3´ (SEQ ID NO: 9); the Leu181 variant, 5´-GGT GAG AAA CAG CAT CAT CAA TAC CAA-3´ (SEQ ID NO: 10); the Leu183 variant, 5´-CAG AAT GGT GAG AAA CAG CAT CAT CAA-3´ (SEQ ID NO: 11). Concurrent amplification of HLA-DP sequence was used as a positive control in each reaction to give a 312 bp band. The primers were: 5´-TCA CTC ACC TCG GCG CTG CAG -3´ (SEQ ID NO: 12) and 5´-CCC TCC CCG CAG AGA ATT AC-3´ (SEQ ID NO: 13). PCR was performed in a Perkin Elmer Cetus DNA thermal cycler using a preliminary cycle (94°C denaturation for 5 min, 60°C annealing for 2 min, and 72°C extension for 2 min) and then 34 cycles (94°C for 2 min, 60°C for 2 min, and 72°C for 2 min). Amplification products underwent electrophoresis in 4% agarose gels before ethidium staining and scoring by two independent observers. Note: careful purification of genomic DNA was essential for effective ARMS testing.

Protocol.

Genotyping and phenotyping were carried out randomised and double blind. The atopy phenotype was ascribed prior to DNA analysis. Freshly extracted DNA samples from all subjects were coded in random order, obscuring all family links. The ARMS testing was performed in duplicate with positive and negative controls. The presence of Leu181 was tested and confirmed by DNA sequencing in the 10 families.

(i) In the random patient sample (Table 1),

Leu181 was found in 25 of 163 individuals (15%) of whom one was homozygous; none showed a Leu183 substitution. Associations were found between the presence of Leu181 and high total serum IgE [odds ratio (OR) 3.07 (95% Confidence Interval 1.25-7.55, Fisher's statistic (FS) = 5.96,p=0.01] and positive IgE tests to grass pollen antigen [OR 2.61 (95% CI 1.07-6.4), FS 4.48, p=0.03] but not to house dust mite antigen (OR 1.44, 95% CI 0.6-3.5). Thirteen (56%) of the Leu181 positive subjects were designated atopic (12 by positive RAST tests) and showed a mean total serum IgE of 300 kU L^{-1}; total serum IgE varies with age, race and other variables but the upper limit of normal, by association with allergen sensitization and allergic symptons, is estimated to be about 100 kU L^{-1} in non-smoking adults in Western populations.

(ii) The results from the 60 nuclear families are shown in Fig. 2. Ten (17%) of the families were found to have the Leu181 variant segregating; this was confirmed by DNA sequencing. In each family, Leu181 was maternally inherited (FS=22.2, p<0.0001). Amongst the children, Leu181 showed a strong association with atopy (all 12 children with Leu181 were atopic; whereas 10 of 12 Leu181 negative children were not non-atopic, FS=18.4, p<0.0001). Atopy was observed in a child without Leu181 in families 2 and 10 and in each instance the father also had atopy without Leu181. Eight of the 10 Leu181 heterozygous mothers (from the various parts of England and Wales) were themselves atopic. DNA was available from both maternal grandparents in two families; Leu181 was of grandmaternal origin where the Leu181 mother was atopic and of grandpaternal origin where the Leu181 mother was non-atopic. Inheritance of Leu181 from a mother is highly predictive of atopy in these ten families, all thirteen such individuals were atopic.

The phenotype in these family subjects was of marked atopy. Only 2 of 14 atopic children showed elevation of total IgE without allergen specific responses (Table 2) and many of the probands had hay fever and eczema in addition to asthma.

Example 3

A study was carried out to examine the prevalence of Leu181 and Leu181/183 in an Australian general population sample. The aim was to test if, when maternally inherited, the variants endowed a significant risk of atopy.

Subjects.

The study population consisted of 1004 subjects in 232 nuclear families from the rural coastal town of Busselton, 200 miles from the main population centre of Perth in South-Western Australia. Families were identified through adults aged 55 or under, from an electoral roll of approximately 9,000. It was emphasised that people who considered themselves normal were important to the study. However, there is known to be a high prevalence of atopy in Bussleton and other Western Australian populations.

Clinical Protocol.

Testing took place in the autumn and winter of 1992, over the three months of May, June and July. A respiratory questionnaire, based on the American Thoracic Society questionnaire but including questions on rhinitis and allergies, was administered. Skin prick testing to common allergens (Dermatophagoides pteronyssinus (HDM), rye grass, cat and dog dander, aspergillus fumigatus, alternaria alternata and negative control (Dome-Hollister-Steir, Spokane USA)) was carried out as previously described (4): wheal

diameters were calculated minus the negative control. Bronchial responsiveness to methacholine was carried out as described (23, 24): the maximum dose administered was 12 µmol. The provocative dose to produce a 20% fall in the FEVI (PD20) was estimated by linear interpolation of points on the dose-response curve. Blood was taken by venipuncture for IgE assays, eosinophil and white cell counts, and DNA studies.

Serology for IgE and white cell counts.

The total serum IgE and specific IgE to whole Dermatophagoides pteronyssinus and Phleum pratense was determined (Pharmacia CAP system FEIA, Sweden). A specific IgE RAST class 1 (≥ 0.35 KU/L) was considered positive. Eosinophil and white cell numbers were estimated by automatic counter (Western Diagnostic Laboratories, Western Australia).

DNA Testing.

DNA was obtained from peripheral blood leucocytes by phenol/chloroform extraction. FcεRI-β Leu181 detection was carried out by the Amplification Refractory Mutation System (ARMS) PCR (25) with the following oligonucleotide primers.

a) 5FU: TGT ATG TGT CAC TTT AAA AGG ACT GGT CAG (SEQ ID NO: 14).

b) 5WK: TTG TCA TTT GTT GCT GTT CAA TAG GAA GTT (SEQ ID NO: 15).

c) 3M: AAT GGT GAG AAA CAG CAT CAT CAT TAC CAA (SEQ ID NO: 16).

d) 3FU: TAA CAT ATC AGT CCT ATT ATC CCA ACC CTC (SEQ ID NO: 17).

Genomic DNA samples (0.25-0.30µg) were amplified in a total volume of 50µl containing 0.5µM of oligonucleotide primers 5FU, 3FU and 5WK, 0.1µM of 3M, 200µM dNTPs, 1 x reaction buffer (43mM KCl, 8.6mM Tris-

HCl (pH8.3), 2.5mM $MgCl_2$, 0.008% gelatin) and 2 units DNA Taq Polymerase (Boehringer Mannheim), overlaid with mineral oil. The reaction mixture (40µl) without enzyme was heated to 95°C for 5 min using a thermal cycler (Hybaid) and held at 80°C for the addition of enzyme (2 units of enzyme in 10µl of reaction buffer). Reaction conditions then followed 35 cycles of 94°C for 1 min, 60°C for 2 min, 72°C for 2 min and 1 cycle 72°C for 10 min. Amplified products were separated in a 3% (3:1 LMP agarose: Nusieve) gel containing ethidium bromide and visualised under UV light. Three bands potentially resulted from the primer combinations: 5FU-3FU gave a 459bp control band. 5WK-3FU gave a 353bp band in the presence of the "wild type" Ile 181. 3M-5FU gave a 163bp band in the presence of Leu181.

A member of each family segregating Leu181 was sequenced by the Sanger method to ensure accuracy of the PCR reaction, and to determine if Leu183 was present. The 459bp 5FU-3FU band from the above reaction was taken to second round PCR with the following internal primers 5D: (5´biotinylated) AAG GAC TGG TCA GAT GGT AG (SEQ ID NO: 18) and 3D: GGC TTC TAT CTA CCT TGT TTC (SEQ ID NO: 19). Single strand template was prepared with strepavidin-labelled magnetic beads (Dynal, Oslo, Norway) and direct solid phase sequencing followed with the sequencing primer 3GS: TCC TTT GAG TTC TTC CCC A (SEQ ID NO: 20).

Genotyping was carried out without knowledge of phenotype and vice versa.

Statistical Analysis

Differences between subjects with different FcεRI-β genotypes were estimated non-parametrically by the Mann-Whitney U test and by Kruskal-Wallis one way ANOVA (SPSS program, McGraw Hill Co., USA). Contingency table analysis, Common Odds Ratios and 95%

Confidence intervals were estimated by exact methods (STATXACT program, Cytel Corp., USA).

Results

Five hundred and two subjects were male. The parents ages were between 30 and 55 years (mean age 40.2, standard deviation (SD) 4.98) and the children between 5 and 27 (mean age 12.6, SD 4.73). Forty-five % of the parents and 43% of the children had a positive skin prick test ≥ 4mm to HDM or rye grass or both; 41% of parents and 44% of children had positive specific IgE titres (RASTs) to either HDM or grass pollen or both. Twenty-three % of the parents and 24% of the children reported wheezing or whistling from their chest in the previous year, and 8% of the parents and 14% of the children reported an attack of asthma in the same interval. Fifty % of the parents and 42% of the children reported episodic sneezing.

The assay for Leu181 failed to amplify in 5 individuals (0.5%). Of the remaining 999 subjects, 45 (4.5%) were positive for Leu181. Twenty-one of these were children; 8 (in 7 sibships) had inherited the variant paternally, and 13 (in 7 sibships) maternally. Sequencing of an individual from each family showed that in each case Leu181 was accompanied by Leu183, so that only the Leu181/Leu183 polymorphism was found in this population.

The 13 children who had inherited Leu181/Leu183 maternally were all atopic (Table 3a). Eleven had symptoms of wheeze or rhinitis or both, and a twelfth, who denied symptoms, had previous physician-diagnosed and treated asthma. Compared to the 531 other children in the population, the 13 had significantly elevated skin tests and RASTs to HDM and to grass pollen (Table 4a). The common odds ratio (OR) for a positive skin test ≥ 4mm to HDM or grass or both,

compared to other children, was 7.6 (95% confidence interval (95%CI) 1.62 - 70.8, p=0.002). The 95%CI for the OR of a positive RAST to either or both allergens was 3.1 - ∞ (p=0.001). When compared only to children with skin tests ≥ 4mm or positive RASTs or both, children with maternal Leu181/Leu183 still had greater skin tests and RASTs to HDM (p=0.005 and p=0.035 respectively).

In addition to measures of the IgE response, the eosinophil counts in the 13 children were significantly above the counts of the other children in the population, and the PD20 to methacholine was significantly lower (Table 4a). Seven children had increased bronchial responsiveness, defined as a PD20 ≤ 10μmol methacholine (23) (OR 3.75, 95%CI 1.06-14.8, p=0.014). Although the trend was for the total serum IgE to be elevated (p=0.08), the IgE levels were not significantly different from other children.

The 8 children who had inherited Leu181/Leu183 paternally were, by contrast, non-atopic, with negative skin tests and RASTs (Table 3b). Their skin tests, RASTs and eosinophil counts were significantly lower than those of other children (Table 4b).

Analysis of variance by ranks showed that maternal Leu181/Leu183, paternal Leu181/Leu183, and other children formed significantly different groups for skin tests to HDM (p=0.0000) or grass (p=0.01), or RASTs to HDM (p=0.003) or grass (p=0.01, and eosinophil counts (p=0.007).

Table 1

Associations between measures of total and specific IgE (RAST) to house dust mite (HDM) and grass pollen and the presence of Leu181 in a random sample of 163 patients

Phenotype		Leu181 -	Leu181 +	Fisher´s statistic	p	Odds ratio (95% confidence interval)
Total Serum IgE	>100	30	11	5.96	0.01	3.07(1.25-7.55)
	<100	109	13			
RAST to HDM	+	46	10	0.73	ns	1.44(0.60-3.50)
	-	93	14			
RAST to Grass Pollen	+	34	11	4.48	0.03	2.61(1.07-6.40)
	-	105	13			

Table 2

The phenotype of members of ten families segregating Leu181

ID	Sex	Atopy status[a]
1.1	M	N
1.2*	F	A

Table 2 (continued)

The phenotype of members of ten families segregating Leu181

ID	Sex	Atopy status[a]
1.3*P	F	A
1.4	F	N
2.1	M	A
2.2*	F	A
2.3*P	M	A
2.4	M	N
2.5*	M	A
2.6	M	A
3.1	M	A
3.2*	F	A
3.3	M	N
3.4*P	F	A
4.1	M	N
4.2*	F	A
4.3	M	N
4.4	M	N
4.5*P	M	A
5.1	M	N
5.2*	F	N
5.3*P	F	A
5.4	F	N
6.1	M	N
6.2*	F	A
6.3*P	F	A
6.4	F	N
7.1	M	A
7.2*	F	A
7.3*P	M	A

Table 2 (continued)

The phenotype of members of ten families segregating Leu181

ID	Sex	Atopy status[a]
7.4	F	N
8.1	M	N
8.2*	F	A
8.3	M	N
8.4*P	M	A
9.1	M	A
9.2*	F	Unknown
9.3*P	M	N
9.4*	F	A
10.1	M	A
10.2*	F	A
10.3	M	N
10.4*P	M	A
10.5	M	A

The phenotype of families are shown in Fig.2. Individuals are numbered from left to right, beginning with the parents.

*, Heterozygotes for FcεRI-β Leu181; P, Proband.
[a]A, Atopic; N, non-atopic.

Table 3a

Clinical details of children with maternally inherited *FcεRI-β Leu181/Leu183*

Pedigree	age	sex	spt[§] HDM *mm*	spt grass *mm*	RAST HDM	RAST grass	Total IgE *IU/L*	PD20[1] *μmol*	eosinophils $10^9/L$	wheeze	asthma	hay fever
6	17	f	5	5	4	3	92	NR	0.54	n	n	y
6	7	m	5	0	5	0	201	7.21	1.63	y	y	y
29	20	f	6	11	5	4	243	NR	0.58	n	n	y
29	18	f	7	0	3	0	63	8.87	0.01	n	n	y
29	14	f	7	5	4	2	166	0.19	0.44	n	y	n
61	8	f	17	0	5	0	215	1.94	1.10	y	y	y
61	14	m	8	8	5	3	550	3.18	0.70	y	y	y
95	11	1	6	4	4	1	178	6.67	0.59	n	n	y
95	10	1	3	2	4	2	137	0.14	0.66	y	y	n
162	14	2	9	4	2	2	15	NR	0.42	y	n	y
181	8	1	6	3	1	2	88	NR	0.19	y	y	n
181	7	1	4	3	2	2	235	2.5	0.23	n	n	y
209	17	2	3	0	0	1	70	NR	0.18	n	n	n

[§]spt= skin prick test
[1]NR = not reactive to maximum dose of methacholine

Table 3b

Clinical details of children with paternally inherited *FcεRI-β Leu181/Leu183*

pedigree	age	sex	spt[§] HDM *mm*	spt grass *mm*	RAST HDM	RAST grass	Total IgE	PD20[¶] *µmol*	eosinophils $10^9/L$	wheeze	asthma	hay fever
103	5	f	0	0	0	0	162	2.66	0.32	n	n	n
141	13	f	0	0	0	0	44	NR	0.05	y	n	n
150	10	f	0	0	0	0	131	1.31	0.30	y	y	y
157	6	f	0	0	0	0	117	NR	0.25	n	n	n
171	14	m	0	0	0	0	6	NR	0.10	n	n	y
171	12	f	0	0	0	0	30	NR	0.04	-	n	-
203	21	m	0	0	0	0	8	NR	0.19	n	n	n
214	19	m	0	0	0	0	80	NR	0.02	n	n	n

[§]spt= skin prick test
[¶]NR = not reactive to maximum dose of methacholine

Table 4a

Mean ranks of measures of atopy in children with maternally inherited FcεRI-β Leu181/Leu183 compared to other children. A high rank indicates a high relative value for a particular parameter.

Parameter	Mann-Whitney U Test Mean Rank		Z	P
	Maternal *Leu181/Leu 183* (n=13)	Others (n=531)		
spt HDM	456.19	273.21	-4.363	0.0000
spt Grass	353.23	270.52	-2.145	0.03
RAST HDM	423.15	268.81	-3.925	0.0001
RAST Grass	343.88	270.75	-1.812	ns
Total IgE	347.5	270.15	-1.756	ns
Eosinophils	356.27	261.67	-2.212	0.03
PD20	196.31	278.43	-2.183	0.03

Table 4b

Mean ranks of paternally inherited *FcεRI-β Leu181/Leu183* compared to other children.

Parameter	Mann-Whitney U Test Mean Rank		Z	P
	Paternal *Leu181/Leu 183* (n=8)	Others (n=536)		
spt HDM	136.00	273.03	-2.635	0.008
spt Grass	165.00	267.54	-2.150	0.03
RAST HDM	159.00	270.66	-2.270	0.02
RAST Grass	146.00	270.86	-2.472	0.01
Total IgE	230.63	269.07	-0.697	ns
Eosinophils	141.63	261.35	-2.245	0.02
PD20	287.38	269.23	-0.390	ns

References

1. Dombrowicz, D., Flamand, V., Brigman, K.K., Koller, B.H. & Kinet, J.-P. Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor α chain gene. Cell 75, 969-976 (1993)

2. Cookson,W.O.C.M., Sharp,P.A., Faux,J.A. & Hopkin,J.M. Linkage between immunoglobulin E responses underlying asthma and rhinitis and chromosome 11q. Lancet i, 1292-1295(1989)

3. Young,R.P. et al. Confirmation of genetic linkage between atopic IgE responses and chromosome 11q13. J.med.Genet.29,236-238(1992)

4. Cookson,W.O.C.M. et al. Maternal inheritance of atopic IgE responsiveness on chromosome 11q. Lancet 340, 381-384(1992)

5. Sandford,A.J. et al. Localisation of atopy and β-subunit of high affinity IgE receptor (FcϵR1) on chromosome 11q. Lancet 341, 332-334(1993)

6. Moffatt,M.F., Sharp, P.A., Faux, J.A., Young, R.P., Cookson, W.O.C. & Hopkin, J.M. Factors confounding genetic linkage between atopy and chromosome 11q. Clin.Exp.Allergy 22, 1046-1051 (1992).

7. Magnusson, C.G. Cord serum IgE in relation to family history and as predictor of atopy disease in earl infancy. Allergy 43, 241-251 (1988).

8. Arshad, S.H., Matthews, S., Gant, C. & Hide, D.W. Effect of allergen avoidance on development of allergic disorders in infancy. Lancet 339, 1493-1497 (1992).

9. Halonen, M., Stern, D., Taussig, L.M., Wright, A., Ray, C.G. & Martinez, F.D. The predictive relationship between serum IgE levels at birth and subsequent incidences of lower respiratory illnesses and eczema in infants. Am. Rev. Respir. Dis. 146, 866-870 (1992).

10. Lympany, P., Welsh, K.I., Cochrane, G.M., Kemeny, D.M. & Lee, T.H. Genetic analysis of the linkage between chromosome 11q and atopy. Clin.Exp. Allergy 22, 1085-92 (1992).

11. Hizawa, N. et al. Lack of linkage between atopy and locus 11q13. Clin.Exp. Allergy 22, 1065-1069 (1992).

12. Rich, S.S., Roitman-Johnson,B., Greenberg, B., Roberts,S & Blumenthal, M.N. Genetic analysis of atopy in three large kindreds: no evidence of linkage to D11S97. Clin.Exp. Allergy 22, 1070-1076 (1992).

13. Amelung, P.J. et al. Atopy and bronchial hyperresponsiveness: exclusion of linkage to markers on chromosomes 11 and 6p. Clin. Exp. Allergy 22, 1077-1084 (1992).

14. Shirakawa, T. et al. Linkage between atopic IgE responses and chromosome 11q in Japanese families. Clin. Genet. (in the press).

15. Collee, J.M., de Vries, H.G. & Gerritsen, J. Allele sharing on chromosome 11q 13 in sibs with asthma. Lancet 342, 936 (1993).

16. Charmley, P., Nguyen J., Tedder, T.F. & Gatti, R. A frequent human CD20 (B1) differentiation antigen DNA polymorphism detected with Mspi is located near 11q12-13. Nucl.Acids Res. 18, 207(1990).

17. Kuster, H., Zhang,L, Brini,A.T., MacGlashan,D.W.J. & Kinet, J.P. The gene and cDNA for the human high affinity immunoglobulin E receptor β chain and expression of the complete human receptor. J.biol.Chem. 267, 12782-12787(1992).

18. Ferrie, R.M. et al. Development, multiplexing, and application of ARMS tests for common mutations in the CFTR gene. AM.J.hum.Genet. 51, 251-262 (1992).

19. Metzger, H. The high affinity receptor for IgE on mast cells. Clin.Exp.Allergy 21, 269-279 (1991).

20. Varin-Blank, N. & Metzger, H. Surface expression of mutated subunits of the high affinity mast cell receptor for IgE. J.biol. Chem. 265, 15685-15694 (1990).

21.Ra, C., Jouvin, M.H.E. & Kinet, J.P. Complete structure of the mouse mast cell receptor for IgE (FcϵR1) and surface expression of chimeric receptors (rat-mouse-human) on transfected cells. J.biol. Chem. 264, 15323-15327 (1989).

22. Kinet, J.P., Blank, U., Ra,C., White, K., Metzger, H. & kochan, J. Isolation and characterisation ofcDNAs coding for the beta subunit of the high affinity receptor for immunoglubulin E. Proc. natn.Acad.Sci. U.S.A. 85, 6483-6487 (1988).

23. Yak, K., Salome, C., Woolcok, A.J. Rapid method of measuring bronchial responsiveness. Thorax. 38. 760-5(1983).

24. Cookson, W.O.C.M., de Klerk, N.H., Ryan, G.R., James, A.L., Musk, A.W. Relative risks of bronchial hyper-responsiveness associated with skin-prick test responses to common antigens in young adults. Clin.Exp. Allergy 21, 473-79 (1991).

25. Newton, C.R. et al. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). NAR 17, 2503-2516 (1989).

26. Oksenberg, D. et al. A single amino-acid difference confers major pharmacological variation between human and rodent 5-HT_{18} receptors. Nature 360, 161-163 (1992).

27. Holt, P.G., McMenamin, C., Nelson, D. Primary sensitisation to inhalant allergens during infance. Pediatr Allergy Immunol 1. 3-13 (1990).

28. Arshad, S.H., Matthews, S., Grant, C., Hide D.W. Effect of allergen avoidance on development of allergic disorders in infancy. Lancet 339. 1493-97 (1992).

29. Beinborn, M., Lee, Y.-M., McBride, E.W., Wuinn, S.M. & Kopin, A.S. A single amino acid of the cholecystokinin-B/gastin receptor determines specificity for non-peptide antagonists. Nature 362, 348-350 (1993).

30. Backer, V et al. Distribution of serum IgE in children and adolescents aged 7 to 16 years in Copenhagen, in relation to factors of importance. Allergy 47, 484-489 (1992)

31. Cline, M.G. & Burrows, B. Distributions of allergy in a population sample residing in Ruscon, Arizona. Thorax 44, 425-431(1989).

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(ii) TITLE OF INVENTION: DIAGNOSTIC METHOD AND THERAPY

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Glu Leu Val Leu Met
  1               5
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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  1               5
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(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

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(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

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(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

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(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

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(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TCACTCACCT CGGCGCTGCA G 21

(2) INFORMATION FOR SEQ ID NO: 13:

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(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

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(2) INFORMATION FOR SEQ ID NO: 14:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

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(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

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(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

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(2) INFORMATION FOR SEQ ID NO: 17:

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(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

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(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GGCTTCTATC TACCTTGTTT C 21

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

TCCTTTGAGT TCTTCCCCA 19

CLAIMS

1. A method of diagnosing atopy or a predisposition to atopy in an individual, which method comprises demonstrating the presence of a mutation or polymorphism in a specific DNA sequence of a gene encoding the beta-subunit of the high affinity IgE receptor in the individual.

2. A method as claimed in claim 1, wherein the gene is on chromosome 11q.

3. A method as claimed in claim 2, wherein the specific DNA sequence is located near the commencement of exon 6 of the gene.

4. A method as claimed in any one of the claims 1 to 3, wherein the specific DNA sequence containing the mutation or polymorphism comprises

5´ GAA TTG GTA TTG ATG (SEQ ID NO: 2) or
5´ GAA TTG GTA GTG ATG (SEQ ID NO: 4)

commencing at nucleotide 5640, or a relevant portion thereof.

5. A method as claimed in any one of claims 1 to 4, comprising amplification of the specific DNA sequence or a relevant portion thereof.

6. A method as claimed in claim 5, wherein the amplification refractory mutation system (ARMS) PCR technique is used.

7. A method as claimed in claim 5, wherein amplification is by PCR, and the amplification products are probed with a sequence-specific nucleic acid probe capable of annealing to a relevant portion of the amplified specific DNA sequence.

8. A method as claimed in any one of claims 1 to 7, performed on a sample of DNA.

9. As new chemical compounds, nucleic acids comprising the sequence

5´ GAA TTG GTA TTG ATG (SEQ ID NO: 2) or

5´ GAA TTG GTA GTG ATG (SEQ ID NO: 4),

or complementary DNA or RNA.

10. A nucleic acid comprising a first portion which corresponds substantially to the whole or part of exon 6 of the gene encoding the beta-subunit of the high-affinity receptor for IgE, which first portion includes one of the following sequences:

5´ TTG GTA TTG or

5´ A TTG GTA GTG (SEQ ID NO: 6) or

5´ TTG GTA GTG A (SEQ ID NO: 7)

or complementary DNA or RNA, and optionally a second portion which corresponds substantially to the whole or part of an intron adjacent to said exon or complementary DNA or RNA.

11. A probe comprising a nucleic acid according to claim 9 or claim 10, linked to a signal moiety or immobilised on a surface.

12. A probe comprising a nucleic acid corresponding substantially to the whole or part of exon 6 of the gene encoding the beta-subunit of the high-affinity receptor for IgE, which nucleic acid includes the following sequence:

5´ ATT GTA GTG,

or complementary DNA or RNA, linked to a signal moiety or immobilised on a surface.

13. The peptide corresponding to a variant of exon 6 of the gene encoding the high affinity IgE receptor on chromosome 11q, and phosphorylation and glycosylation products, and characteristic fragments thereof.

14. The peptide claimed in claim 13, comprising the amino acid sequence:

Glu Leu Val Leu Met (SEQ ID NO: 3) or

Glu Leu Val Val Met (SEQ ID NO: 5),

or a relevant portion thereof.

15. Antibodies to the peptides, phosphorylation and glycosylation products, and characteristic fragments, according to claim 13 or 14, and fragments thereof.

16. A method as claimed in claim 1, using antibodies according to claim 15 to identify a protein variant corresponding to the specific DNA sequence.

Figure 1

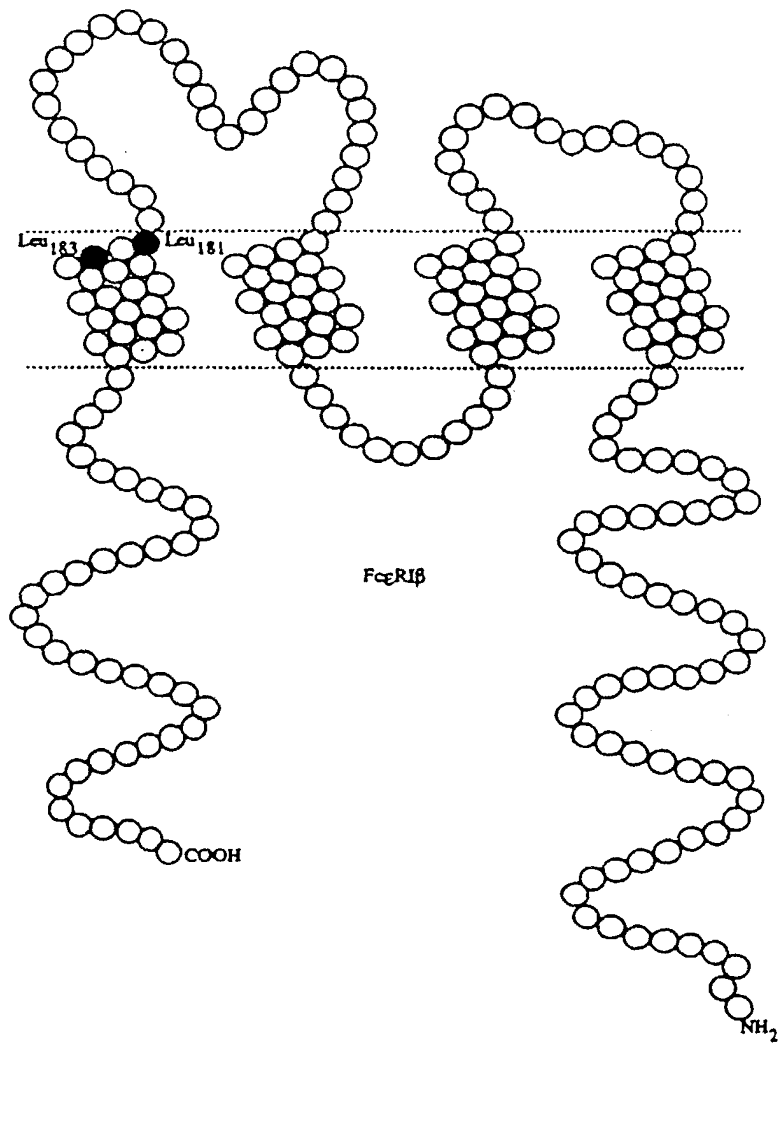
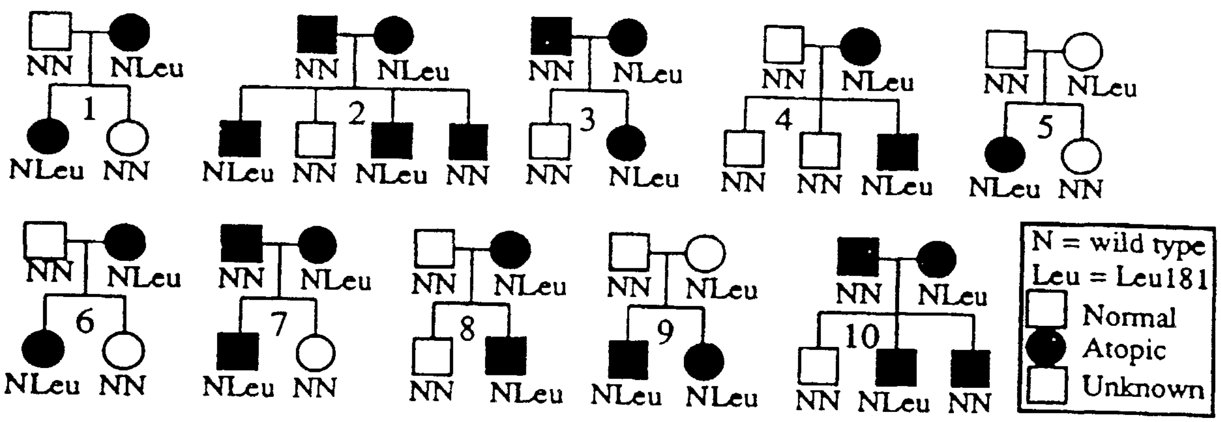


Figure 2


N = wild type
Leu = Leu181
Normal
Atopic
Unknown
NN
NLeu
1
2
3
4
5
6
7
8
9
10

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/01801

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07H21/04 C07K14/705 C07K16/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Geneseq Database entry R14770 Accession number R14770; 3 February 1992 Descriptor Field: Beta subunit of high affinity IgE receptor abstract ---	13,14
X	JOURNAL OF BIOLOGICAL CHEMISTRY., vol.8, 25 May 1986, BALTIMORE US pages 6765 - 71 HOVE-JENSEN, B. ET AL 'Phosphoribosylphosphate synthetase of Escherichia coli' especially residues 719-733 see page 6771; figure 4 --- -/--	13,14

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
22 December 1994	30.12.94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL Database entry CEZC84 Accession Number Z19157; 27 December 1992 Sulston, J. et al: C. Elegans Sequencing & Nature 356:37-41, 1992 abstract ---	10
X	MOLULAR ENDOCRINOLOGY, vol.4, no.2, 1990, BALTIMORE US pages 235 - 244 GOLDSTEIN, B. ET AL 'The rat insulin receptor' see figure 1C especially residues 3300-3312 ---	10
X	THE LANCET, vol.341, 6 February 1993, UK pages 332 - 34 SANDFORD, A. ET AL 'Localisation of atopy and beta-subunit of high-affinity IgE receptor (Fc eta-RI) on chromosome 11q.' cited in the application	1,2
A	see the whole document ---	3-14,16
A	THE LANCET, vol.340, 15 August 1992, UK. pages 381 - 84 COOKSON, W. ET AL 'Maternal inheritance of atopic IgE responsiveness on chromosome 11q' cited in the application see the whole document ---	1-14,16
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol.267, no.18, 25 June 1992, US pages 12782 - 87 KUSTER, H. ET AL 'The gene and cDNA for the human high affinity immunoglobulin E receptor beta-chain and Expression of the complete human receptor.' cited in the application -----	

2

International application No.

INTERNATIONAL SEARCH REPORT

PCT/GB94/01801

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

See Annex

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Claims searched completely: 1-14, 16
Claims searched incompletely: 15

The definition of the peptide fragments against which antibodies are sought for protection is so vaguely defined that a comprehensive search is not possible. The search was thus limited to antibodies against the beta subunit of the high-affinity IgE receptor in general.